ANDROLOGY

Destruction of Protamine in Human Sperm Inhibits Sperm Binding and Penetration in the Zona-Free Hamster Penetration Test but Increases Sperm Head Decondensation and Male Pronuclear Formation in the Hamster–ICSI Assay

ALI AHMADI[1] and SOON-CHYE NG[1,2]

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Purpose: *Our purpose was to investigate the fertilizing ability of human protamine-damaged sperm in a heterologous system using hamster oocytes.*
Methods: *The protamine of the sperm were damaged by exposure to dithiothreitol, a disulfide-reducing agent. Their ability to penetrate and form male pronuclei were investigated using the zona-free hamster penetration test and the hamster–intracytoplasmic sperm injection assay, respectively.*
Results: *The zona-free hamster penetration test revealed that protamine-damaged sperm are unable to bind and penetrate the hamster oocyte. On the other hand, hamster–intracytoplasmic sperm injection assay results showed that 56.9% and 39.2% of the injected oocytes developed male pronuclei in protamine-damaged and live-intact sperm groups, respectively, with a significant difference in these rates ($P < 0.01$).*
Conclusions: *This study shows that protamine-damaged sperm are able to undergo sperm head decondensation and male pronuclear formation only when injected into the ooplasm, although they cannot bind and penetrate through the zona and enter the ooplasm.*

KEY WORDS: protamine-damaged; dead sperm; binding; penetration; sperm head decondensation; male pronuclear formation.

[1] Department of Obstetrics and Gynecology, National University of Singapore, Lower Kent Ridge Road, Singapore 119074, Singapore.
[2] To whom correspondence should be addressed.

INTRODUCTION

During fertilization, the egg cytoplasm must reduce the strong intra- and interprotamine disulfide bonds to enable sperm nuclear decondensation with subsequent male pronuclear formation (1,2). The reversal of condensation and stabilization of mammalian sperm chromatin are necessary for the transcription of sperm genetic information after fertilization resulting in the initiation of DNA synthesis (3).

Under in vitro conditions, decondensation of eutherian nuclei can be induced by treatment with a disulfide-reducing agent alone (4,5) or reducing agents in combination with neutral detergents (6,7), anionic detergents (8–10), proteases (11,12), or salts (13,14). The need for a reducing agent to elicit in vitro decondensation of most species suggests that the reduction of disulfide bonds may be essential for the ability of the chromatin to disperse. Sperm nucleoproteins have been shown to degrade enzymatically when sperm are incubated in vitro with a reducing agent alone or in combination with a neutral detergent (15). Protamine-damaged sperm are unable to bind and penetrate the zona pellucida of guinea pig oocytes and enter the ooplasm (16,17). It is not clear whether this statement can be applied to the human. Are only prefertilization steps affected, or is the fertilization process (sperm head decondensation and male pronuclear formation) also inhibited? The objective of this study is to investigate the prefertilization (sperm binding, penetration) and fertilization (sperm head decondensa-

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tion, male pronuclear formation) ability of human protamine-damaged sperm in a heterologous system, using hamster oocytes.

MATERIALS AND METHODS

Preparation of Spermatozoa

Frozen–thawed human semen samples from proven donors were used for this study. The sperm were processed by the discontinuous Percoll gradient (40%, 70%, 90%) separation method (18).

Induction and Evaluation of Damage to the Nuclear Protein (Protamine) of the Sperm

DTT (dithiothreitol), a disulfide-reducing agent, was used to break the disulfide bonds of the protamine in the sperm. The sperm were incubated with 5 m*M* DTT in protein-free medium for 30 min and then washed several times to remove the excess DTT.

Acridine orange (AO) staining was used to evaluate protamine integrity of the sperm. Ten microliters of sperm suspension was mixed with 20 μl of a low-pH detergent solution (0.15 *M* NaCl, 0.08 *N* HCl, 0.01% Triton X-100, pH 1.4). After 30 sec, 60 μl of AO staining solution (6 μg/ml AO in 0.2 *M* Na_2HPO_4, 1 m*M* disodium ethylenediaminetetraacetic acid, 0.15 *M* NaCl, 0.1 *M* citric acid monohydrate, pH 6) was added, and 10 μl of sample was placed on the slide and covered with a coverslip. Approximately 250 sperm per sample were evaluated by fluorescence microscopy. The nuclei of sperm with intact protamine fluoresced green (AO associated with double-strand DNA), while those with damaged protamine fluoresced orange–red (AO associated with single strand DNA).

Preparation of Oocytes

Six- to eight-week-old golden hamsters on day 1 of the estrous cycle (with vaginal discharge) were stimulated with intraperitoneal injections of 30 IU pregnant mare's serum gonadotropin (Folligon; Intervet International B.V., Boxmeer, Holland) followed 56 hr later with 30 IU human chorionic gonadotropin (Chorulon; Intervet International B.V.). The recovery of oocytes and details of the hamster–intracytoplasmic sperm injection (ICSI) assay and zona-free hamster penetration tests have been described elsewhere (19,20).

Microinjection Procedure

The holding and microinjection pipettes were made by drawing glass capillary tubes with a horizontal Sutter puller (P-87; Sutter Instruments, USA) and were further processed on a Narishige microgrinder (EG-4; Narishige, Tokyo) and De Fonbrune microforge (MF-1; Technical Products International Inc., St. Louis, MO). The injection pipettes had an internal diameter of 5–6 μm, an outer diameter of 7–9 μm, and a bevel angle of 40°, with a sharp spike. The holding pipettes had an outer diameter of 80 μm and an inner diameter of 40 μm.

Microinjection and oocyte holding systems were controlled with a simple pneumatic system (18). They were connected to ordinary 3-ml syringes via Teflon tubing which joined to the pipette holder. ICSI was carried out on the 37°C heated stage using a Zeiss motorized micromanipulator and an Axiovert 135 inverted microscope as described previously (19).

Evaluation of Oocytes and Culture of Embryos

In the zona-free hamster penetration experiment, after 3 hr of incubation the oocytes were washed four times in medium to remove loosely adherent sperm and fixed in 1% glutaraldehyde. They were then stained with aceto-orcein and examined under × 100 magnification. The number of sperm penetrated was counted and the penetration rate and index were calculated.

In the hamster–ICSI experiment, 18 to 20 hr after injection they were checked for male pronuclei formation and fixed in 1% gluteraldehyde, stained with aceto-orcein, and examined for sperm head decondensation and male pronuclei formation under × 100 magnification. The state of the sperm nucleus within the cytoplasm of oocyte was classified into six stages (21), which briefly follow: stage a, intact sperm nucleus; stage b, decondensing sperm nucleus; stage c, complete decondensed sperm nucleus; stage d, round sperm nucleus enclosed within a nuclear membrane; stage e, an enlarged nucleus with nucleoli; and Ab, abnormal decondensation.

Statistic Analysis

All statistical analysis was carried out using the *t* test at a 5% level of significance. The calculations were made using the SPSS software package.

Table I. The Effect of Protamine Damage on Penetration of Human Sperm into Hamster Oocytes

Sperm	No. of hamster oocytes	Penetration rate (%)	Penetration index
Protamine-damaged	227	0	0
Live–intact	233	71.2	2.95

RESULTS

In order to damage the nuclear protein of the sperm, they were exposed to DTT, a disulfide-reducing agent, to break the disulfide bonds; the damage was evaluated by AO staining. In the experimental group, 100% of the sperm showed red fluorescence after staining with AO, confirming that this treatment caused damage to the protamine of all sperm. The fertilizing ability of protamine-damaged human sperm was studied by investigating their ability to penetrate and form male pronuclei in hamster oocytes using the zona-free hamster penetration test and the hamster–ICSI assay, respectively. The zona-free hamster penetration results revealed that damage to the protamine of the human sperm inhibits their binding as well as penetration to the hamster oocyte. Table I summarizes the results of experiments in which the sperm suspension was exposed to DTT prior to insemination. In the control group, 71.5% of the sperm were able to penetrate the zona-free oocytes. The penetration index, which is defined as the number of penetrated sperm per the total number of oocytes, was 2.95 for live–intact sperm. On the other hand, 0% of the DTT-treated sperm were able to penetrate the zona-free oocyte. Whether protamine-damaged sperm have the ability to undergo pronuclear formation when they are injected into oocytes was then investigated (Table II). Sperm head decondensation and male pronuclear formation were seen in 91.2% and 56.9% of the injected oocytes, respectively, in the experimental group (Table III), while these rates were significantly lower, at 59.6% and 39.2%, respectively, in the control group ($P < 0.001$).

Table III. Sperm Head Decondensation and Male Pronuclear Formation Following Injection of Live–Intact and Protamine-Damaged Human Sperm into Hamster Oocytes

Sperm	Oocytes (%) with	
	Male pronuclei	Decondensed sperm head*
Protamine-damaged	56.9	91.2
Live–intact	39.2	59.6

* Significantly different ($P < 0.001$).

DISCUSSION

In mammalian spermiogenesis, the nuclear histone and histone-like proteins that are found in the early spermatid nucleus are replaced with a series of more basic proteins until, in the mature spermatid nucleus, only protamine remains (22–25). Protamine in mammalian sperm is a low molecular weight protein, rich in arginine and cysteine. As mature testicular spermatids pass into the caput epididymides, free sulfhydryl groups on the cysteine residues of protamine begin to oxidize to form disulfide bonds. This process continues during passage through the epididymides, and in consequence, the mature cauda sperm have nuclei highly stabilized by protamine disulfide bonds (8,26).

In the sperm nucleus, according to a model proposed by Balborn (26) and Ward and Coffey (27), the protamines bind to DNA by lying lengthwise inside the minor groove, and DNA strands are packaged side by side in a linear array. Intramolecular and intermolecular covalent disulfide bondings of protamines make chromatin very stable. Disruption of these bridges is a prerequisite for decondensation of the fertilizing sperm nucleus (1).

Decondensation, in turn, is a prelude to protamine replacement by histones and subsequent reactivation

Table II. Results of Intracytoplasmic Sperm Injection of Live–Intact and Protamine-Damaged Human Sperm into Hamster Oocytes

Sperm	No. of replicates	No. of oocytes		Sperm head decondensation stage[a]					
		Injected	Cultured	e	d	c	b	a	Ab
Protamine-damaged	10	216	195	111	30	22	15	10	7
Live–intact	10	212	191	75	9	12	18	73	4

[a] Stage a, intact sperm head; stage b, decondensing sperm head; stage c, completely decondensed sperm head; stage d, the sperm nucleus is round and the nuclear membrane has formed; stage e, the nucleus enlarges and nucleoli have appeared; Ab, abnormal decondensation.

of the sperm genome in the oocyte (28). Perreault *et al.* (29) have demonstrated that, following microinjection into the hamster oocyte, condensed hamster spermatid nuclei, which have relatively few disulfide bonds, decondensed more rapidly than cauda epididymal sperm nuclei, which are rich in disulfide bonds. In other words, the greater the extent of protamine disulfide bonding, the more time is required for oocyte reducing factors to initiate decondensation.

To investigate the fertilizing ability (penetration, sperm head decondensation, male pronuclear formation) of protamine-damaged human sperm, they were exposed to DTT prior to the zona-free hamster penetration test and the hamster–ICSI assay. DTT reduces both intra- and inter-protamine disulfide bonds to produce sulfhydryl groups (26). On incubation with DTT, decondensation is observed to begin in the postacrosomal region of the nucleus, proceeding rostrally and then distally to include the entire nucleus (2). The observations are supported by transmission electron microscopy studies that reveal chromatin fibrils extending from a condensed mass in the postacrosomal region of the nucleus, reflecting a similar morphology of the sperm nucleus during the early stages of fertilization (30). The AO staining test, a test for evaluation of nuclear protein integrity, was used to confirm the disulfide-bond destruction. This cytochemical method allows the differentiation between double-stranded (green fluorescence) and single-stranded (red fluorescence) DNA because of the metachromatic properties of AO (31–33). Our experiment shows that protamine-damaged human sperm are unable to fertilize as they have lost their binding ability to the oocyte. However, if this step is bypassed, i.e., by injection into the cytoplasm of an oocyte, they are able to undergo sperm head decondensation and male pronuclear formation. Whether they have the ability to continue embryonic and fetal development remains to be investigated. Fleming *et al.* (16) have shown that DTT blocks the binding of acrosome-reacted guinea pig sperm to the egg plasma membrane of hamster oocytes. Yanagimachi *et al.* (17) demonstrated that DTT blocks not only binding of acrosome-reacted guinea pig sperm to oolemma of homologous species, but also binding to the zona pellucida. DTT-treated spermatozoa coiled repeatedly with zona of zona-intact eggs or vitellin of zona-free eggs but did not bind to the zona or vitelline surface. However, though a few spermatozoa remained on the zona and vitelline surfaces for a short time (up to 1 min), they eventually detached and swam away. They have also shown that DTT inhibits both capacitation and acrosome reaction of guinea pig spermatozoa. They concluded that DTT appears to inhibit a variety of important steps in the fertilization process, at least in the guinea pig. In this experiment we have demonstrated that DTT will inhibit the prefertilization process only up to sperm entry to the oocyte. In fact, the treated sperm, if injected into the oocyte, will result in significantly higher decondensation and pronuclear formation rates compared with controls.

Failed fertilization is encountered regularly in human in vitro fertilization programs. Among other factors, this could be due to damage to sperm protamine. Therefore, performing the AO staining test together with the standard semen analysis tests is recommended. Where there is damage to the nuclear proteins, patients should be put into an ICSI program. Protamine-damaged sperm have the capacity to undergo sperm head decondensation and male pronuclear formation only when injected into the cytoplasm of an oocyte, although they cannot bind and penetrate through the zona and enter the oolemma. The embryonic development of resultant embryos remains to be investigated.

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